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(57) Abstract

The subject invention relates to recombinant immunotoxins, and to uses thereof. In particular, the invention relates to two immunotoxins, referred to as B3(Fv)-PE40 and B3(Fv)-PE38KDEL, which may be used in the treatment of mammalian cancer.

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RECOMBINANT IMMUNOTOXINS

The present application is a continuation-in-part of U.S. patent application serial number 07/596,289 filed on October 12, 1990 and hereby incorporated in its entirety by reference.

BACKGROUND OF THE INVENTION

Technical Field

The subject invention relates to recombinant immunotoxins, and to uses thereof. In particular, the invention relates to two immunotoxins, referred to as B3(Fv)-PE40 and B3(Fv)-PE38KDEL, which may be used in the treatment of mammalian cancer.

BACKGROUND INFORMATION

The monoclonal antibody B3 (MAb B3) is a recently isolated murine antibody which is directed against a carbohydrate antigen in the LE^y family (Pai et al., Proc. Natl. Acad. Sci. USA 88:3358-62 (1991)). This antigen is found on the surface of many mucinous carcinomas of the colon, stomach, ovaries, breast, lung as well as some epidermal carcinomas. Because it reacts with only a limited number of normal tissues, MAb B3 is an ideal candidate for the treatment and diagnosis of cancer. In order to create a cytotoxic agent, MAb B3 has been previously chemically coupled to two different forms of Pseudomonas exotoxin (PE) (U.S. patent 4,545,985). One of these is the full length toxin (PE) and the other a truncated derivative (PE40) (Kondo et al., J. Biol. Chem. 263:9470-75 (1988) & Pai et al., supra). Both of these immunotoxins have been shown to be selectively cytotoxic to tumor

cells that contain the B3 antigen on their surface, and these immunotoxins have also been shown to cause complete tumor regression in mice bearing human tumor xenografts (Pai et al., Proc. Natl. Acad. Sci. USA 88:3358-62 (1991)). Although these first generation immunotoxins have properties that indicate they should be developed further as drugs for the treatment of cancer, immunotoxins made by chemical conjugation methods have several undesirable properties. For example, the chemical modifications can change the antibody and affect its binding to the antigen. Furthermore, the purified immunotoxins are a heterogeneous mixture of antibody-toxin molecules connected to each other via different positions on the antibody and the toxin. Thus, PE can be coupled either to the light- or heavy-chain of the antibody and to different positions on each of these chains.

The second generation of immunotoxins are made as recombinant antibody Fv-fusion proteins in bacteria. It has been shown that single chain antigen binding proteins (scAB's, scFv's) made from the Fv portions of the heavy and light chain of antibodies held together by a polypeptide linker can have the same binding properties as their full length two chain counterparts (Bird et al., Science 242:423-26 (1988) and Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-83 (1988)). Furthermore, fusion proteins composed of scAB's linked to toxins retain the binding capacity of the scAB as well as the activity of the toxin (Chaudhary et al., Nature 339:394-97 (1989); Batra et al., J. Biol. Chem. 265:15198-202 (1990); Batra et al., Proc. Natl. Acad. Sci. USA 86:8545-49 (1989); Chaudhary et al., Proc. Natl. Acad. Sci. USA 87:1066-70 (1990)).

Potent single chain immunotoxins have been made previously by fusing the Fv domains of

antibodies directed at the interleukin 2 receptor (Chaudhary et al., Nature 339:394-97 (1989) & Batra et al., J. Biol. Chem. 265:15198-15202 (1990)) or at the transferrin receptor (Batra et al., Proc. Natl. Acad. Sci. USA 86:8545-49 (1989)) to truncated forms of PE or diphtheria toxin (Chaudhary et al., Proc. Natl. Acad. Sci. USA 87:9491-94 (1990)). Receptors often make good immunotoxin targets because they are cell surface proteins that can be rapidly internalized, and toxins must be internalized in order to kill cells.

Copending U.S. patent application serial number 07/341,361, filed on April 21, 1989, describes a recombinant immunotoxin comprising an antibody-PE40 recombinant single chain fusion protein. This immunotoxin has been shown to have a cytotoxic effect on cells possessing particular antigens or receptors.

The immunotoxins described herein are selectively cytotoxic to cultured mammalian tumor cells bearing the B3 antigen and also cause regression of mammalian tumors *in vivo*. These immunotoxins are described in detail below.

* * * * * * *

All U.S. patents and publications referred to herein are hereby incorporated by reference.

SUMMARY OF THE INVENTION

The present invention relates to two novel immunotoxins which may be used for the treatment of mammalian cancer.

In particular, the present invention encompasses a recombinant DNA molecule comprising:

(i) a DNA segment which encodes the Fv region of both the light and heavy chains of a monoclonal antibody; and

(ii) a vector for introducing the DNA segment into host cells, the vector comprising a gene which encodes an altered form of a bacterial toxin or a portion of the toxin. The altered form of the toxin retains translocating and enzymatic activity.

Again, the vector utilized in creating the recombinant molecule may be, for example, pULI1, pULI3, a derivative of either of these plasmids, or any other vector which allows for the expression of recombinant proteins in microorganisms. The bacterial toxin referred to above is Pseudomonas exotoxin.

A derivative of a plasmid or gene would include changes in the DNA sequence of the plasmid or gene, respectively, that do not eliminate either the antigen binding of the Fv fragment or the translocating and enzymatic activity of the toxin.

The present invention also includes a host cell stably transformed with the recombinant DNA molecule of in a manner allowing expression of the immunotoxin encoded by the recombinant DNA molecule. The host cell may be a procaryotic cell such as, for example, an Escherichia coli cell.

Furthermore, the present invention also relates to a recombinantly produced immunotoxin consisting of a toxin protein or a portion thereof and the Fv region of the light and heavy chains of a monoclonal antibody. Once again, the toxin utilized is Pseudomonas exotoxin. The monoclonal antibody utilized is MAb B3.

The present invention also encompasses a composition comprising the immunotoxin described directly above and a pharmaceutically acceptable carrier.

The invention also includes a method of treating cancer in a patient comprising

administering, to the patient, an amount of the composition described-above, sufficient to effect the treatment.

The immunotoxins of the present invention are created by:

(i) cloning a DNA sequence which encodes the Fv region of both the heavy and light chain of a monoclonal antibody into a vector, the vector comprising a gene which encodes an altered form of a bacterial toxin or a portion of the toxin; and

(ii) transforming a host cell with the resulting vector of step (i), thereby allowing for expression of the immunotoxin.

Again, the altered form of the toxin retains translocating and enzymatic activity.

The monoclonal antibody which may be utilized in the production of the immunotoxin is MAbB3. This monoclonal antibody preferentially reacts with human tumors.

The immunotoxin which can be produced is selected from the group consisting of B3(Fv)-PE40 and B3(Fv)-PE38KDEL, or derivatives thereof. For example, B3(Fv)-PE38KDEL can be created as a derivative of B3(Fv)-PE40 by deleting sequences in the Ib domain of B3(Fv)-PE40 and by changing the carboxy terminus of the molecule to increase the cytoxicity thereof. Furthermore, these two steps and/or other alterations can be carried in forming other derivatives.

The vector used in the method of forming the immunotoxin may be, for example, pULI1, pULI3, a derivative of either of these plasmids, or any other vector that allows for the expression of recombinant proteins in microorganisms. The bacterial toxin, referred to above, is <u>Pseudomonas</u> exotoxin (PE).

It should be noted that, prior to the present invention, no single chain immunotoxin had

been shown to have an antitumor effect in a mammal. Furthermore, the anti-tumor effect of the present immunotoxins is quite remarkable as complete tumor regression may be exhibited in only a few days with only a small amount of the relevant immunotoxin being required. Such a result requires a molecule which can enter the tumor, effectively bind to the tumor cells, and subsequently kill these cells. Thus, the molecule must penetrate into the interior of the tumor cells. It is thought that the immunotoxins of the present invention possess such an ability or such significant anti-tumor effects would not otherwise be observed.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 represents the strategy for the cloning of the heavy and light chain Fv genes of MAb B3 and the construction of expression vectors (e.g., plasmids) for the expression of B3(Fv) immunotoxins. The cloning strategy is a variation of that previously described (Chaudhary et al., Proc. Natl. Acad. Sci. USA 87:1066-70 (1990)). The plasmid pVC38H, which is used as a vector for construction of immunotoxins from heavy and light chain Fv regions, contains an NdeI and a HindIII recognition sequence preceding the PE40 gene (Chaudhary et al., *supra* (1990)). The sequences of the PCR primers are shown in Example I. (*) indicates a PCR-generated mutation and was repaired by site directed mutagenesis; (L) indicates the region encoding the $(Gly_4Ser)_3$ linker which serves to join heavy and light chains of the immunotoxin.)

FIGURE 2 shows the nucleotide sequences encoding the heavy and light chain Fv region of MAb B3. (a) - the heavy chain Fv coding region extends from position 30 to 383, the light chain Fv gene

from position 433 to 767 and the linker from 384 to 432. The deduced amino acid sequence is shown in plain letters; below in italic letters is the protein sequence determined by Edman sequencing of the antibody. The first amino acid encoded by the cloned heavy chain Fv gene is Asp instead of Glu due to the oligonucleotide primer used, at position 456-465 is the region where the PCR cloning artifact was repaired. This sequence encodes the same amino acids as the original B3 light chain gene but uses other codons. Homology comparisons to the known nucleotide sequence of PAC1 Ig kappa chain (Taub et al., J. Biol. Chem. 264:259-65 (1989)) which is most homologous to the B3 light chain indicates that the original sequence was most probably CTCTCCCTG instead of TTGAGTTTA. Thus the natural B3 light chain gene has a sequence repetition 5'(CCAGTCT[CC)ACTCTCC]3' between positions 445 and 465 which is responsible for the incorrect primer annealing in PCR. (b) - sequence at the 3'-end of the light chain for expression of the single chain B3(Fv) alone. (SD) - Shine Dalgarno consensus sequence; (*) - translation stop signal. (Term) - transcription terminator.

FIGURE 3 shows the recombinant B3(Fv) and B3(Fv) immunotoxins from bacterial inclusion bodies. SDS-PAGE (24) (12.5%) showing (a) total cell protein of induced bacteria producing single chain B3(Fv); (b) total protein of cells producing B3(Fv)-PE40; (c,d) supernatant of sonicated cells producing (c) B3(Fv) or (d) B3(Fv)-PE40; (e) inclusion bodies containing B3(Fv); (f) inclusion bodies containing B3(Fv)-PE40 ; (g) purified B3(Fv)-PE40 protein after gel filtration. MW: molecular weight standard.

FIGURE 4(a) represents the toxicity of B3(Fv)-PE38KDEL on different cell lines. Cytotoxicity assays were performed as described in

Example IV. (b): Inhibition of the cytotoxicity of B3(Fv)-PE38KDEL by MAb B3. Competition by MAb B3 was perf rmed on A431 cells as described in Example IV.

FIGURE 5 shows blood levels of B3(Fv)-PE38KDEL in mice. Balb/c mice were injected i.v. with 10 μg of B3(Fv)-PE38KDEL and immunotoxin levels measured at different time periods. Bars indicate the standard deviation.

FIGURE 6 represents the effect of B3(Fv)-PE38KDEL on the growth of A431 tumors in nude mice. Mice were injected with $3x10^6$ A431 cells on day 0 and treated beginning on day 4 with i.v. injections every 12 hrs x 6. A: (○) untreated; (●) 10 μg B3(Fv)-PE38KDEL; B: (□) 2.5 μg B3; (■) 5 μg B3(Fv)-PE38KDEL; C: (△) 2.5 μg anti-Tac (Fv)-PE38KDEL; (▲) 2.5 μg B3(Fv)-PE38KDEL; (--○--) 0.5 μg B3(Fv)-PE38KDEL; D: treatment began on day 7 with i.v. injections every 12 hrs x 8. (○) untreated, (■) 5 μg B3(Fv)-PE38KDEL. Bars = standard deviation.

DETAILED DESCRIPTION OF THE INVENTION

In order to prepare the immunotoxins of the present invention, initially sequences encoding the heavy and light chain Fv domains of the murine monoclonal antibody B3 are cloned. As mentioned above, this MAb recognizes an antigen present on many carcinoma cells, and may be useful for the treatment of various types of cancers (Pai et al., Proc. Natl. Acad. Sci. USA 88:23358-62 (1991). The heavy and light chain regions of MAb B3 are then connected by a flexible linker (Gly,Ser), which starts at the carboxyl end of the heavy chain Fv domain and ends at the amino terminus of the light chain Fv domain. The resulting gene encodes the

B3(Fv) domain in the form of a single chain antigen binding protein. This B3(Fv) gene is then fused to sequences encoding two different truncated forms of the PE molecule to obtain single chain B3(Fv) immunotoxins. These recombinant immunotoxins can kill carcinoma cells containing the B3 antigen without affecting control cells. The cytotoxicity of the recombinant B3(Fv)-PE40 is similar to a chemical conjugate of B3 and PE40 (B3-LysPE40) (Pai et al., supra (1991)), but B3(Fv)-PE38KDEL is five-fold more active (see Table 1). Thus, less material needs to be administered to patients which results in a better anti-tumor effect, minimal side effects as well as a diminished production of neutralizing antibodies to the recombinant toxin. Moreover, since B3(Fv)-PE38KDEL is much smaller in size than B3-LysPE40, it will penetrate tumors far more effectively.

Furthermore, B3(Fv)-PE38KDEL causes complete regression of A431 tumors grown in immunodeficient mice. This makes the B3(Fv) derived single chain immunotoxins a promising alternative to B3 chemical conjugates and a possible second generation immunotoxin for the treatment of solid tumors. For example, the immunotoxins could be administered either intravenously for cancers which have spread or could also be administered locally (i.e., into the bladder for use in the treatment of bladder cancer or into the peritoneal cavity for use in the treatment of ovarian cancer). The immunotoxins may be given for 7-10 days, for example. The treatment could then be repeated as often as necessary.

The authenticity of the cloned DNA fragments can be proven by comparing the amino terminal protein sequences of the B3 heavy and light chains with the amino acid sequences deduced from

the reading frames of the cloned genes (FIG. 2). The sequences of the cloned Fv coding regions are shown in Figure 2.

The analysis of the cytotoxicity of B3-immunotoxins showed the same sensitivity-pattern of different cell lines towards the recombinant immunotoxins as towards the chemical conjugates. All these immunotoxins were very cytotoxic to carcinoma cells that express the B3 antigen on their surface including MCF7 (breast), A431 (epidermoid), CRL1739 (gastric) and LNCaP (prostate). Thus, all of these cell lines, as well as many others, react with the B3 react antibody. (See Table 1 below.)

Table 1: Activities of B3 immunotoxins on different cell lines

			Cytotoxicity (ID_{50}) in ng/ml (pM)		
Cell Line	Cancer Type	B3 antigen	B3(Fv)-PE40	B3(Fv)-PE38KDEL	B3-LysPE40
MCF7	breast	++	3 (50)	0.2 (3.2)	3 (16)
CRL1739	gastric	++	3 (50)	0.3 (5)	3 (16)
A431	epidermoid vulva	+	3 (50)	0.8 (13)	8 (42)
LNCaP	prostate	+	40 (1330)	20 (325)	85 (460)
KB3-1	epidermoid cervix	-	>1000	>1000	>1000
HUT102	adult T cell leukemia	-	>1000	>1000	>1000

Also, the recombinant single chain B3-Fv immunotoxins did not affect B3 antigen-negative control cells. The cytotoxicity of the recombinant B3(Fv)-PE40 (ID_{50} = 50 pM; 3.0 ng/ml) was similar to the chemically linked B3-immunoconjugate (ID_{50} = 42 pM; 8 ng/ml), whereas B3(Fv)-PE38KDEL was much more active than the chemical conjugate (ID_{50} 13 pM; 0.8 ng/ml). This is despite the fact that the single chain immunotoxins possess only one antigen binding site per molecule and the chemical conjugate has two (see Table 2 below).

Table 2: Structure and Activity of B3 Immunotoxins on A431 Cells

Immunotoxin	Toxin Part	C-Term	Binding	ID_{50}
B3 chemical conjugate	PE40	REDLK	bivalent	8.0 ng/ml (42 pMol)
B3(Fv) fusion protein	PE40	REDLK	monovalent	3.0 ng/ml (50 pM)
B3(Fv) fusion protein	PE38	KDEL	monovalent	0.8 ng/ml (13 pM)

B3(Fv)-PE38KDEL has two features that distinguish it from B3(Fv)-PE40. One is that a portion of domain Ib encompassing amino acids 365-380 is deleted. This removes the disulfide bond formed between cysteine residues at positions 372 and 379, which might form disulfide bonds with other cysteines during the renaturation process and thereby result in the creation of inactive chimeric toxins. The second feature is that the carboxyl terminus of the toxin is changed from the original sequence REDLK to KDEL. When the disulfide bond was removed in other molecules, the increase in activity was small. For example, TGFα-PE38 is only 50% more active than TGFα-PE40 (see Siegall et al., J. of Biol. Chem. 264:14256-61 (1989)). IL6PE38 is no more active than IL6-PE40. Changing REDLK to KDEL usually only produces a two to three fold increase in activity of chimeric toxins.

B3(Fv)-PE38KDEL was tested for its antitumor effect in nude mice bearing A431 tumors. Complete regression of tumors was observed when mice received 2.5 μg, 5 μg or 10 μg of the chimeric toxin twice daily for three days, despite the fact that B3(Fv)-PE38KDEL has a short lifetime (15-20 min) in the circulation. B3(Fv)-PE38KDEL also produced complete regression of tumors about 1 cm in diameter. Previously, it was found that even the administration of 75 μg per day for 5 days of a

chemical conjugate composed of B3 and PE40 (see Table 2) only produced partial regr ssion of large tumors despite the fact that the chemical conjugate has a much l nger lifetime in the blood (4 hrs.). The recombinant molecule probably has a higher antitumor activity in the mouse model because of its small size which allows better access to tumor cells.

The present invention can be illustrated by the use of the following non-limiting examples:

Example I

Cloning of DNA Fragments Encoding the Heavy and Light Fv Region of MAb B3

Cloning experiments and propagation of plasmids were carried out generally in E. coli HB101 (Boyer et al., J. Molec. Biol. 41:459-72 (1969)). DNA fragments encoding the Fv portions of heavy and light chain of MAb B3 were obtained by (PCR-) amplification of single stranded DNA which was synthesized by random primed reverse transcription of mRNA from a MAb B3 producing hybridoma cell line. Polymerase chain reaction (Saiki et al., Science 239:487-91 (1988)) was performed using the Perkin Elmer GeneAmp kit and an Perkin Elmer/Cetus thermocycler, under conditions as described (Chaudhary et al., Proc. Natl. Acad. Sci. USA 87:1066-70 (1990)). For amplification of the heavy chain Fv coding region, the present inventors chose the primer pair B3-H1 (5'TAACTAGGATCCGTCCATATG GATGTGAAGCTGGTGGAGTCTGG3') and B3-H2 (5'TGGATAGACTG ATGGGGATCCGCCTCCGCCTGAGGAGAC3') and for the light chain B3-L1(5'GTCTCCAAGCTTGGGGATCCGGTGGTGGCGGATCTGG AGGTGGCGGAAGCGATGTGCTGACCCAGTCTCC3') and B3-L2(5'AGTTGGTGCAGCATCAAAAGCTTT[G/T]A[G/T][T/C]TCCAGCT T[T/G]GT]G/C]CC3'). These oligonucleotides have at their 3' end constant sequences that occur at the

beginning and end of mouse Fv DNA. At their 5' end are restriction endonuclease recognition sites (NdeI, BamHI, HindIII) for cloning of the PCR products as shown in FIG. 1. The products of the amplifications of heavy- and light chain Fv DNA fragments were identified by agarose gel electrophoresis to be DNA fragments between 350 and 400 bp. They were purified from gels, cut with BamHI or HindIII (FIG. 1) and after purification on a second gel, ligated with HindIII- or BamHI-linearized and dephosphorylated pBR322 vector (Bolivar et al., Gene 2:95-113 (1977)). The nucleotide sequence of the light- and heavy chain Fv coding region of MAb B3 was determined from double stranded plasmid DNA using sequencing primers (New England Biolabs) adjacent to the BamHI or HindIII site of pBR322 and a T7 polymerase sequencing reagent kit (United States Biochemicals).

Example II

Construction of Plasmids for Expression of B3(Fv) and B3(Fv)-Immunotoxins

The expression plasmid pVC38H contains the gene from the immunotoxin TGFα-PE40 under control of the T7 promoter (Chaudhary et al., Proc. Natl. Acad. Sci. USA 87:1066-70 (1990)), the Tϕ transcription terminator at the 3'-end of the PE40 coding region and the single strand replication origin, F+, to generate single stranded phage DNA by cotransfection with (M13) helper phages, if desired, to create derivatives of the plasmid by site directed mutagenesis. The TGFα coding region in pVC38H has an NdeI recognition site at the 5'-end and a HindIII site at the point of connection to the DNA encoding PE40. To create a plasmid for expression of the immunotoxin B3(Fv)-PE40 (pULEE3), the TGFα gene was

rem ved and replaced by the B3(Fv) gene in a 3-fragment ligation, using an NdeI/BamHI fragment of the heavy chain coding region and the BamHI/HindIII fragment encoding the light chain Fv (FIGURE 1). Because sequence analysis showed a mutation (deletion and frameshift) at the 5' end of the light chain Fv gene due to a sequence repetition in the PCR primer annealing region, site-directed mutagenesis was performed (Kunkel, T.A., Proc. Natl. Acad. Sci. USA 82:488-92 (1985)), using uridine incorporated single stranded phagemid DNA (pULEE3) as the mutagenesis template. In the resulting plasmid (pULI1), the correct amino end of the B3 light chain established by partial protein sequencing of MAb B3, is reconstructed.

To make another B3(Fv) immunotoxin, B3(Fv)-PE38DKEL, the PE40 coding region was removed from pULI1 from the HindIII site to an EcoRI site positioned just beyond the PE40 gene, and replaced by a HindIII/EcoRI fragment from pRK79K encoding the PE variant PE38KDEL which lacks domain Ia (amino acids 1-252) and part of domain Ib (amino acids 365-380), and also contains an altered carboxyl terminal sequence KDEL (Chaudhary et al., Proc. Natl. Acad. Sci. 87:308-12 (1990)). The expression plasmid pULI4 for production of B3(Fv) was constructed by removal of the light chain and PE40 coding region from pULI1 from BamHI to EcoRI which was replaced by a PCR fragment obtained by amplification of the light chain Fv coding sequence with the primer-pair B3-L3 + B3-L4. The primer B3-L3 (5'TTGGGGATCCGGTGGTGGCGGATCTGGA3') is similar to B3-L1, used for cloning of light chain Fv from cDNA and B3-L4 ('AGCGGGAATTCATTATTTAATTTCCAGCTTTGTCCCCGAC3') is in the 3' part for priming the PCR identical to B3-L2, but at the 5' end the HindIII site for fusion

to PE-sequences is replaced by translation stop codons followed by an EcoRI recognition sequence.

Example III

Expression and Purification of Recombinant B3 (Fv) - Immunotoxins

Plasmids were transformed in the expression-host E. coli BL21 (λDE3) (Studier et al., J. Mol. Biol. 189:113-30 (1986)). The bacteria were grown in superbroth containing 2% glucose, 0.05 % $MgSO_4$ and 100 μg/ml ampicillin, induced in the log phase at OD_{600} of 3.0 with 1 mM IPTG and harvested 90 min later. About 30% of the total protein of the induced cultures was the recombinant expression product which was deposited in inclusion bodies. The purified inclusion bodies contained almost pure recombinant protein, which had the expected size of about 67 kDa for a single chain immunotoxin (FIGURE 3). The recombinant immunotoxin molecules were solubilized, refolded, purified, and the protein was analyzed as previously described (Chaudhary et al., Nature 339:394-97 (1989) & Batra et al., J. Biol. Chem. 265:15198-202 (1990)). Protein concentrations were determined by Bradford assay (Bradford, M.M., Anal. Biochem. 72:848-54 (1976)). The purity of the molecules is shown in FIGURE 3.

Example IV

Cytotoxic Activity of Chemically Linked and Recombinant Immunotoxins

Assays measuring inhibition of protein synthesis were previously described (Chaudhary et al., Nature 339:394-97 (1989) & Batra et al., J. Biol. Chem. 265:15198-202 (1990)). All assays were

performed in 96 well plates each well containing 1.6 x 10^4 cells in 200 μl medium. For competition assays designed to prove the specificity of the recombinant immunotoxins, the medium was changed and 50 μg/well of antibody was added 15 min prior to the addition of the immunotoxin.

As shown in Figure 4 and in Table 1, the recombinant single chain immunotoxins inhibited protein synthesis in cells expressing the B3 antigen but not in non-expressing cells, similarly to the previously described results with chemical conjugate of B3 with a truncated form of PE (Pai et al., Proc. Natl. Acad. Sci. USA 88:3358-62 (1991)). The relative potencies of the chemical conjugate and the single chain immunotoxins were about the same on the four antigen positive cell lines MCF-7, CRL 1739, A431 and LNCaP. The most active agent was B3(Fv)-PE38KDEL. To analyze whether the cytotoxicity of B3(Fv)-immunotoxins was specific, competition experiments were carried out with an excess of MAb B3. The data in FIGURE 4b shows that the intoxication of A431 carcinoma cells by B3(Fv)-PE38KDEL is due to the specific binding to the B3 antigen, since its cytotoxicity was blocked by excess MAb B3 but not by MAb HB21 which recognizes the transferrin receptor on these cells (Haynes et al., J. Immunol. 127:347-51 (1981)). A large excess of MAb B3 is necessary for reversal of cytotoxicity, probably because there is a large amount of the B3 antigen on the surface of A431 cells (Pai et al., Proc. Natl. Acad. Sci. USA 88:23358-62 (1991)).

Example V

Assay of Blood Levels of B3(Fv)-PE38KDEL in Mice

Six week old (19-20 gm) female Balb/c mice were injected with 10 μg of B3(Fv)- PE38KDEL in the

tail vein. Blood was drawn at various time intervals and the level of the immunotoxin measured by incubating serum with A431 cells and measuring inhibition of protein synthesis. A standard curve was made with pure B3(Fv)-PE38KDEL and the blood level of immunotoxin (which is shown in Figure 5) calculated using this curve.

Example VI

Anti-Tumor Activity of B3(Fv)-PE38KDEL in Nude Mice Bearing a Human Epidermoid Carcinoma

A431 cells (3×10^6) were injected subcutaneously on day 0 into female nude mice (4-6 weeks old, 18-20 gm). Mice with 5 mm by 5 mm tumors, that usually developed by day 4, were treated with B3(Fv)-PE38KDEL or as a control with MAbB3 or anti-Tac(Fv)-PE38KDEL (Chaudhary et al., Nature 339:394-97 (1989)). Because the lifetime of B3(Fv)-PE38KDEL in the circulation of the mice was observed to be only 15-20 min (FIGURE 5), six injections were given at 12 hour intervals into the tail vein, starting 4 days after tumor implantation. Each treatment group consisted of five animals. The volume of the tumor was calculated by [tumor volume in cm^3=length x $width^2$ x 0.4].

As shown in FIGURE 6, injection of either 2.5, 5 or 10 μg twice daily produced complete tumor regression. Partial regression was observed when only 0.5μg was injected. No toxicity was observed at these doses. In addition, when mice with large tumors about 1 cm in diameter were treated with 5μg twice a day for 4 days, complete regression of these large tumors containing about 5×10^6 cells rapidly occurred (FIGURE 6D). Regression of MCF-7 tumors (breast carcinoma) also was observed with 5μg twice daily of B3(Fv)-PE38KDEL. In control experiments,

mice were treated with either MAb B3 or anti-Tac(Fv)-PE38KDEL, which is cytotoxic to cells with IL2 receptors but n t for A431 cells (Chaudhary et al., supra (1989)) and no antitumor effect was observed.

WHAT IS CLAIMED IS:

1. A recombinant DNA molecule c mprising:

(i) a DNA segment which encodes the Fv region of both the light and heavy chains of a monoclonal antibody; and

(ii) a vector for introducing said DNA segment into host cells, said vector comprising a gene which encodes an altered form of a bacterial toxin or a portion of said toxin.

2. The recombinant DNA molecule of claim 1 wherein said vector is pULI1, pULI3, or a derivative thereof.

3. The recombinant DNA molecule of claim 1 wherein said bacterial toxin is *Pseudomonas* exotoxin.

4. A host cell transformed with the recombinant DNA molecule of claim 1 in a manner allowing expression of an immunotoxin encoded by said recombinant DNA molecule.

5. The host cell of claim 4 wherein said cell is a procaryotic cell.

6. The host cell of claim 5 wherein said procaryotic cell is an *Escherichia coli* cell.

7. A recombinantly produced immunotoxin consisting of a toxin protein or a portion thereof and the Fv region of the light and heavy chains of a monoclonal antibody.

8. The immunotoxin of claim 7 wherein said toxin is Pseudomonas exotoxin.

9. The immunotoxin of claim 7 wherein said monoclonal antibody is MAb B3.

10. A composition comprising the immunotoxin of claim 7 and a pharmaceutically acceptable carrier.

11. A method of treating cancer in a patient comprising administering to said patient an amount of the composition of claim 10 sufficient to effect said treatment.

12. A method of producing an immunotoxin comprising the steps of:

(i) cloning a DNA sequence which encodes the Fv region of both the heavy and light chain of a monoclonal antibody into a vector, said vector comprising a gene which encodes an altered form of a bacterial toxin or a portion of said toxin, said altered form retaining translocating and enzymatic activity; and

(ii) transforming a host cell with the resulting vector of step (i), thereby allowing for expression of said immunotoxin.

13. The method of claim 12 wherein said monoclonal antibody is MAb B3.

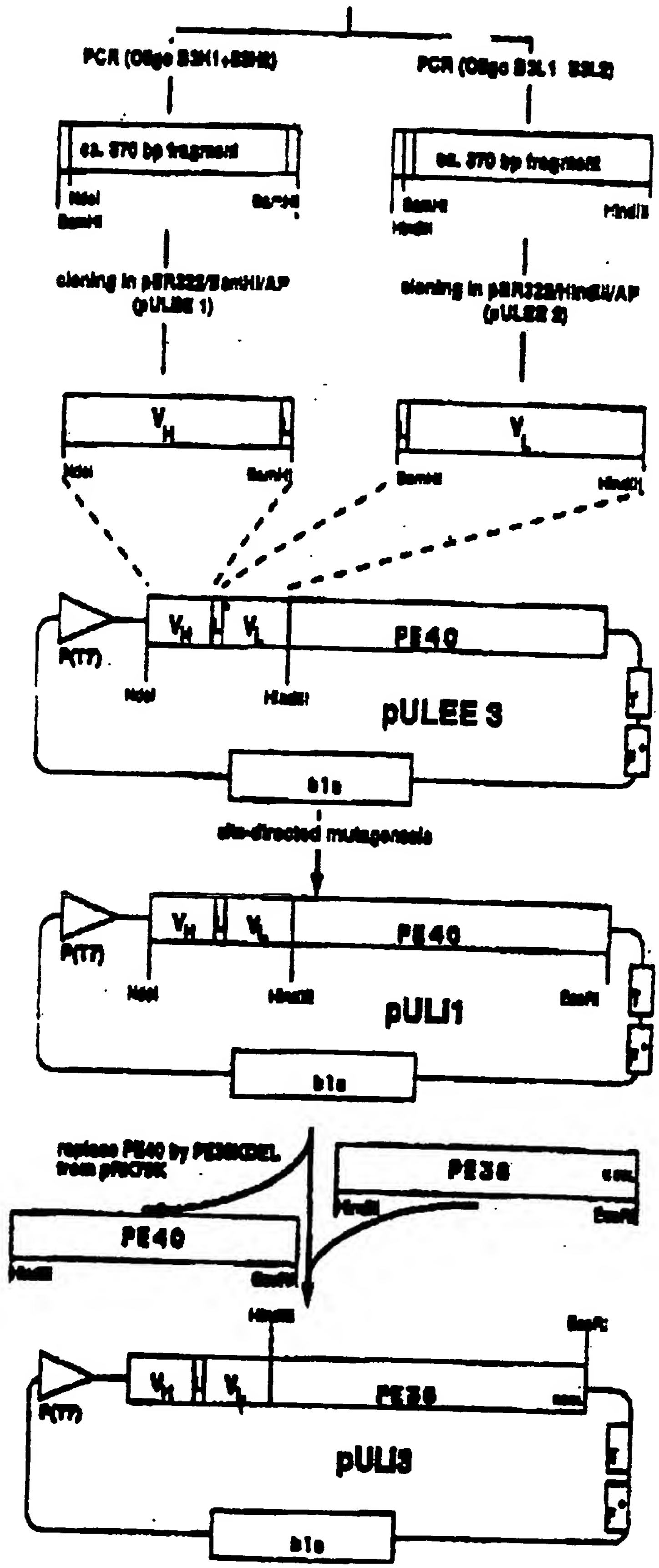
14. The method of claim 12 wherein said monoclonal said preferentially reacts with human tumors.

15. The method of claim 12 wherein wherein said immunotoxin is selected from the group consisting f B3(Fv)-PE40 and B3(Fv)-PE38KDEL.

16. The method of claim 12 wherein said immunotoxin is a derivative of B3(FV)-PE40 or B3(Fv)-PE38KDEL.

17. The method of claim 12 wherein said vector of step (i) is pULI1, pULI3, or a derivative thereof.

18. The method of claim 12 wherein said bacterial toxin is Pseudomonas exotoxin (PE).



PCR (Oligo B3H1+B3H2)
PCR (Oligo B3L1 B3L2)
ca. 370 bp fragment
ca. 370 bp fragment
NdeI
BamHI
BamHI
HindIII
cloning in pBR322/BamHI/AP
(pULEE 1)
cloning in pBR322/HindIII/AP
(pULEE 2)
V_H
V_L
NdeI
BamHI
BamHI
HindIII
V_H
V_L
PE40
P(T7)
NdeI
HindIII
pULEE 3
bla
site-directed mutagenesis
V_H
V_L
PE40
P(T7)
NdeI
HindIII
EcoRI
pULI1
bla
replace PE40 by PE38KDEL from pRK79K
PE38
PE40
HindIII
EcoRI
HindIII
EcoRI
V_H
V_L
PE38
P(T7)
pULI3
bla

Fig. 1 1/6

a

```
                                          NdeI |------Fv heavy chain--------->
  1 TTTAACTTTAAGAAGGAGATATACATATGGATGTGAAGCTGGTGGAGTCTGGGGGAGGCT  60
                SD            M  D  V  K  L  V  E  S  G  G  G  L
                                 E  V  K  L  V  E  S  G  G  G  L

 61 TAGTGCAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAACCTCTGGATTCACTTTCAGTG 120
      V  Q  P  G  G  S  L  K  L  S  C  A  T  S  G  F  T  F  S  D
      V  Q  P  G  G  S  L

121 ACTATTACATGTATTGGGTTCGCCAGACTCCAGAGAAGAGGCTGGAGTGGGTCGCATACA 180
      Y  Y  M  Y  W  V  R  Q  T  P  E  K  R  L  E  W  V  A  Y  I

181 TTAGTAATGATGATAGTTCCGCCGCTTATTCAGACACTGTAAAGGGCCGGTTCACCATCT 240
      S  N  D  D  S  S  A  A  Y  S  D  T  V  K  G  R  F  T  I  S

241 CCAGAGACAATGCCAGGAACACCCTCTACCTGCAAATGAGCCGTCTGAAGTCTGAGGACA 300
      R  D  N  A  R  N  T  L  Y  L  Q  M  S  R  L  K  S  E  D  T

301 CAGCCATATATTCCTGTGCAAGAGGACTGGCCTGGGGAGCCTGGTTTGCTTACTGGGGCC 360
      A  I  Y  S  C  A  R  G  L  A  W  G  A  W  F  A  Y  W  G  Q

                                           BamHI
361 AAGGGACTCTGGTCACTGTCTCCTCAGGCGGAGGCGGATCCGGTGGTGGCGGATCTGGAG 420
      G  T  L  V  T  V  S  S  G  G  G  G  S  G  G  G  G  S  G  G
    <-----Fv heavy chain--|-------------Linker-------------------

    -Linker---|----------------Fv light chain-------------------->
421 GTGGCGGAAGCGATGTGCTGATGACCCAGTCTCCATTGAGTTTACCTGTCAGTCTTGGAG 480
      G  G  S  D  V  L  M  T  Q  S  P  L  S  L  P  V  S  L  G  D
               D  V  L  M  T  Q  S  P  L  S  L  P  V  S  L  G  ?

481 ATCAAGCCTCCATCTCTTGCAGATCTAGTCAGATCATTGTACATAGTAATGGAAACACCT 540
      Q  A  S  I  S  C  R  S  S  Q  I  I  V  H  S  N  G  N  T  Y
      Q

541 ATTTAGAATGGTACCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTT 600
      L  E  W  Y  L  Q  K  P  G  Q  S  P  K  L  L  I  Y  K  V  S

601 CCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCA 660
      N  R  F  S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T

661 CACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAAGGTT 720
      L  K  I  S  R  V  E  A  E  D  L  G  V  Y  Y  C  F  Q  G  S

                                                     HindIII
721 CACATGTTCCATTCACGTTCGGCTCGGGGACAAAGCTGGAAATTAAAGCTTT........ 772
      H  V  P  F  T  F  G  S  G  T  K  L  E  I  K  A  F --> PE40
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b

```
                                                         EcoRI
721 CACATGTTCCATTCACGTTCGGCTCGGGGACAAAGCTGGAAATTAAATAATGAATTCC.. 779
      H  V  P  F  T  F  G  S  G  T  K  L  E  I  K  *  * --> Term
```

Fig. 2

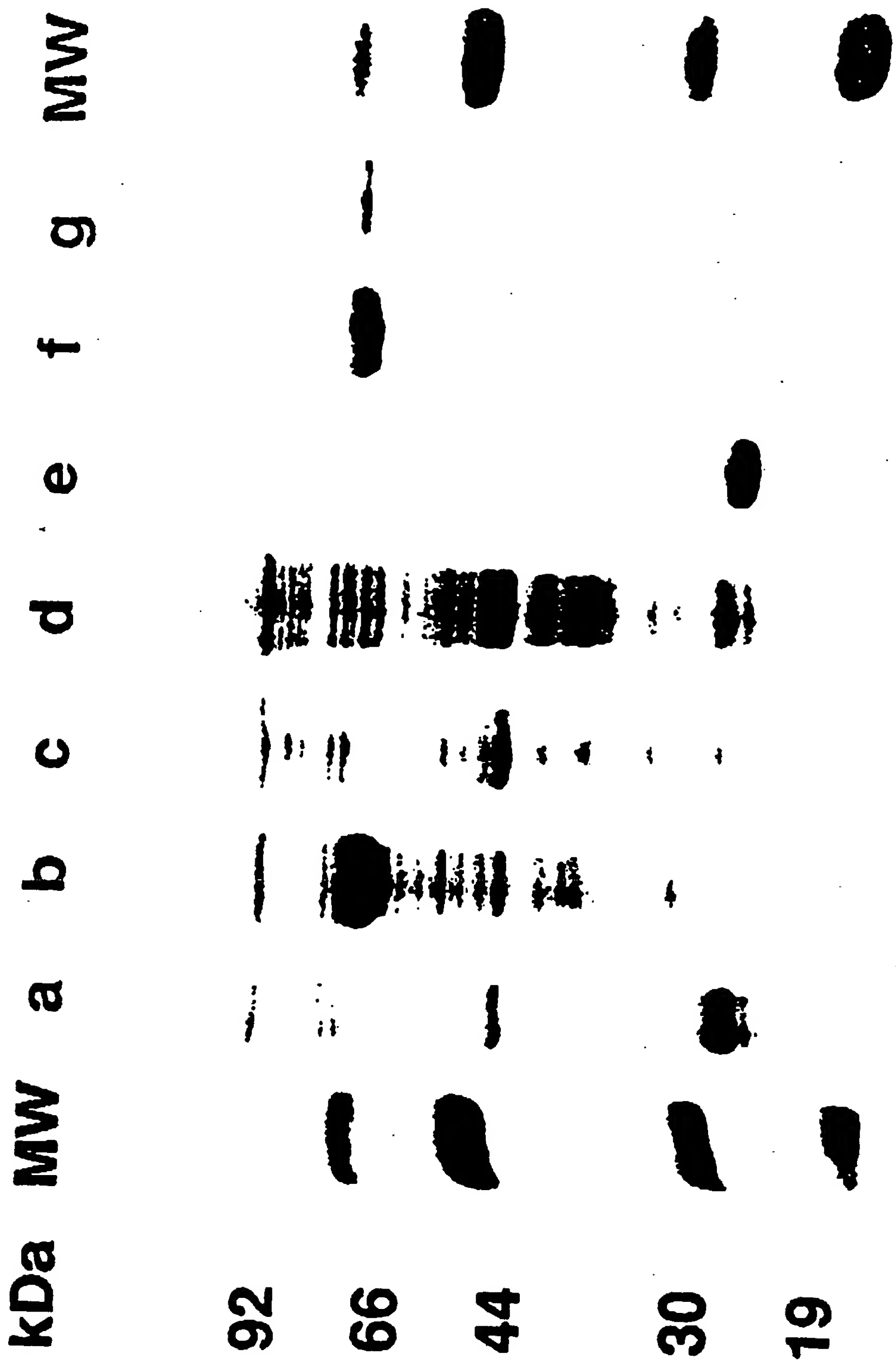
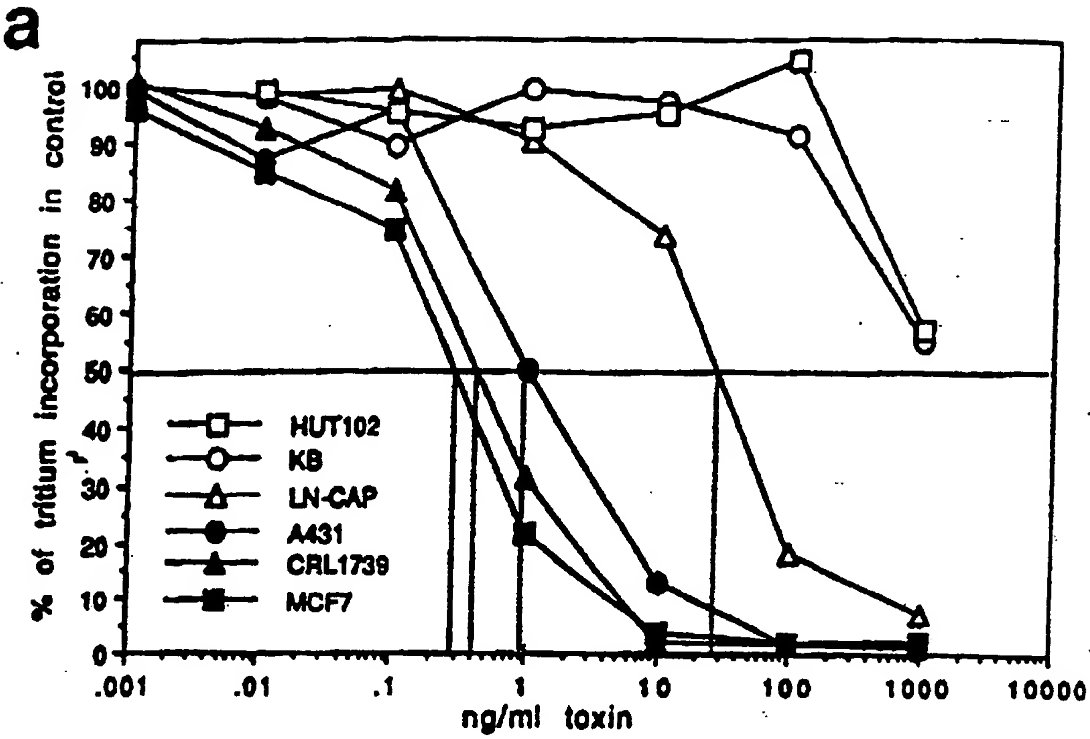


Fig. 3


a
% of tritium incorporation in control
100
90
80
70
60
50
40
30
20
10
0
.001
.01
.1
1
10
100
1000
10000
ng/ml toxin
HUT102
KB
LN-CAP
A431
CRL1739
MCF7

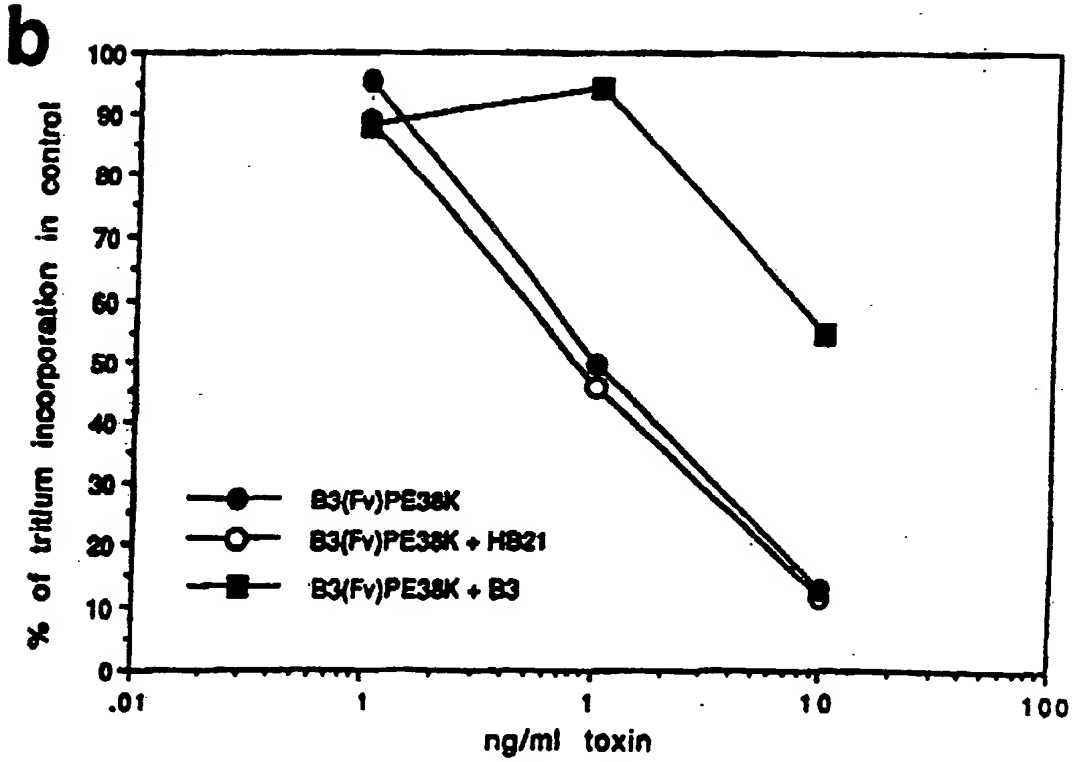

b
% of tritium incorporation in control
100
90
80
70
60
50
40
30
20
10
0
.01
1
1
10
100
ng/ml toxin
B3(Fv)PE38K
B3(Fv)PE38K + HB21
B3(Fv)PE38K + B3

Fig. 4

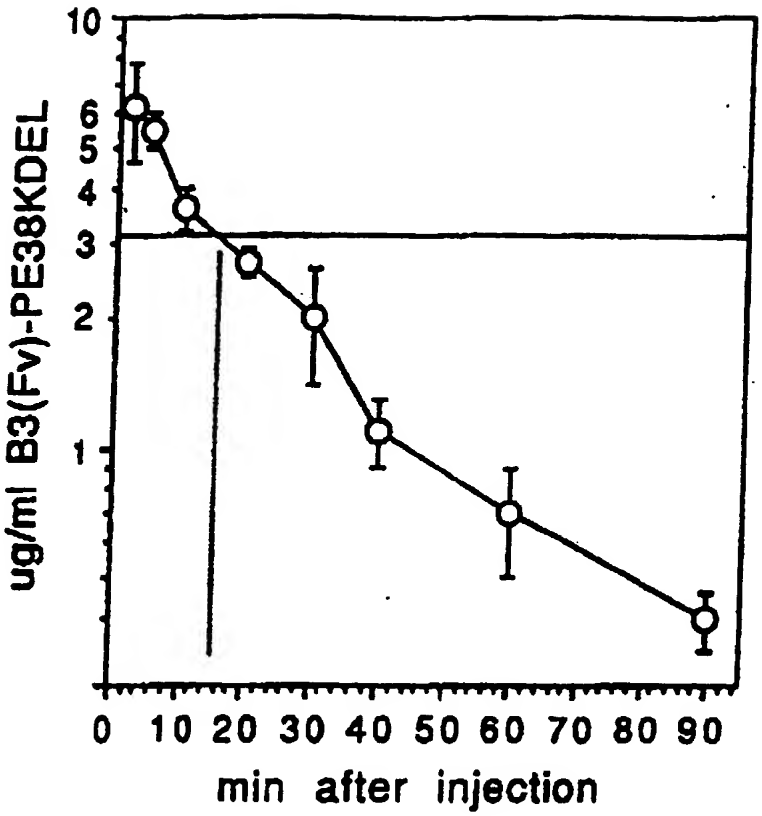

ug/ml B3(Fv)-PE38KDEL
10
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
90
min after injection

Fig. 5

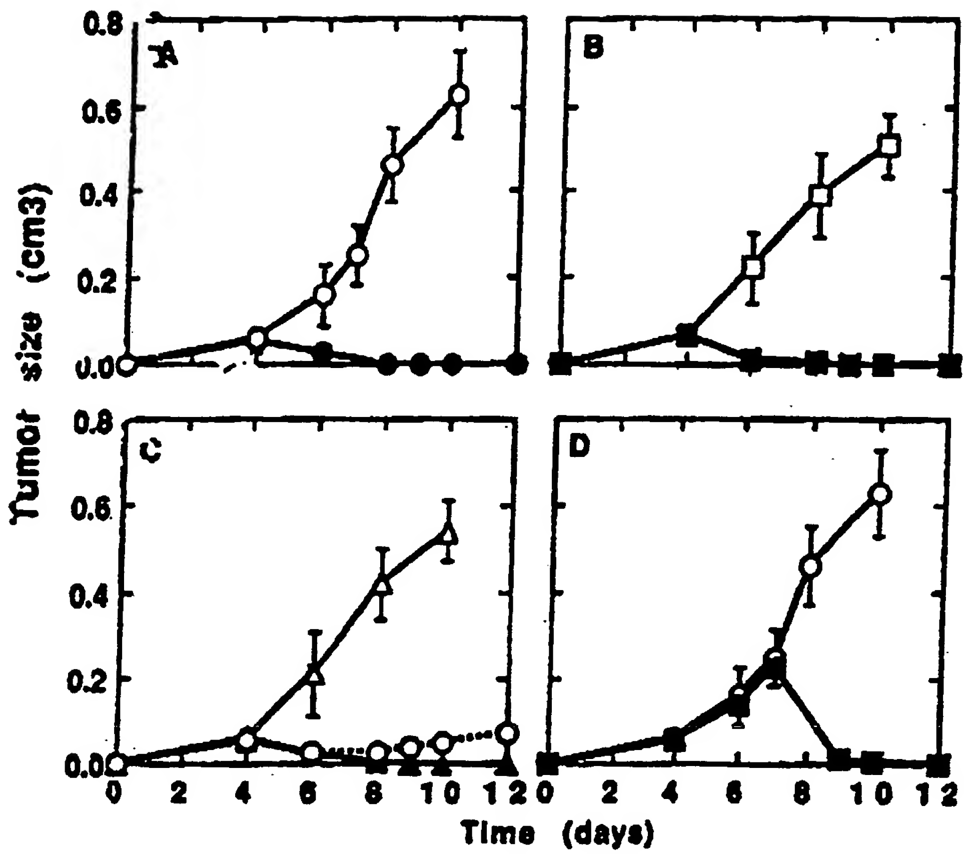


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08257

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :536/27; 435/91, 69.1, 70.21, 172.2, 240.27, 252.3, 320.1; 530/867
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 435/91, 69.1, 70.21, 172.2, 240.27, 252.3, 320.1; 530/867

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE
search terms: Pseudomonas, Fv, B3, immunotoxin, antibody, fusion, exotoxin.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 51, issued 15 July 1991, Ira Pastan et al., "Characterization of Monoclonal Antibodies B1 and B3 that React with Mucinous Adenocarcinomas", pages 3781-3787, see entire document.	1-18
Y	Journal of Biological Chemistry, Volume 263, No. 19, issued 05 July 1988, Toshihiko Kondo, et al., "Activity of Immunotoxins constructed with Modified Pseudomonas Exotoxin A lacking the Cell Recognition Domain", pages 9470-9475, see entire document.	1-18
Y	Journal of Biological Chemistry, Volume 265, No. 25, issued 05 September 1990, Janendra K. Batra, et al., "Anti-Tac(Fv)-PE40: A Single Chain Antibody Pseudomonas Fusion Protein Directed at Interleukin 2 Receptor Bearing Cells", pages 15198-15202, see entire document.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1992	17 DEC 1992

Name and mailing address of the ISA/	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DONALD E. ADAMS, PH.D.
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08257

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 87, issued 01 February 1990, Vijay K. Chaudhary et al., "A rapid method of cloning functional variable-region antibody genes in Escherichia coli as single-chain immunotoxins", pages 1066-1070, see entire document.	1-18
Y	Nature, Volume 339, No. 6223, issued 01 June 1989, Vijay K. Chaudhary et al., "A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin", pages 394-397, see entire document.	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08257

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12P 19/34, 21/06, 21/02, 15/00, 5/00, 1/02= C07K 3/00, 13/00, 15/00, 17/00; A61K 35/14, C07H 15/02, 17/00

Form PCT/ISA/210 (extra sheet)(July 1992)*